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N. O'Connor

For and on behalf of RWS Group plc

The 5th day of December 2000

Novel defective adenoviruses and corresponding complementation lines

The invention relates to novel defective adenoviruses permitting the transfer and expression of genes of interest to a host eukaryotic cell or organism, as well as to new complementation lines complementing *in trans* the essential viral functions which have been deleted from the genome of these recombinant adenoviruses. The invention is of very special interest for prospects of gene therapy, in particular in man.

Adenoviruses are DNA viruses which display a broad host range. They have been demonstrated in many animal species and many cell types. There are many serotypes which differ in particular in respect of their genome sequence. Most human adenoviruses are only marginally pathogenic and generally produce only benign symptoms.

The adenovirus enters the permissive host cell via a specific receptor, and it is then internalized and passes into endosomes. Their acidification contributes to a change in conformation of the virus and to its emergence into the cytoplasm. The viral DNA associated with certain viral proteins needed for the first steps of the replicative cycle then enters the nucleus of the infected cells, where its transcription is initiated by cellular enzymes. Replication of the adenoviral DNA takes place in the nucleus of the infected cells and does not require cell replication. Assembly of the new virions also takes place in the nucleus. In a first stage, the viral proteins assemble so as to form empty capsids of icosahedral structure, in which the adenoviral DNA is then encapsidated. The viral particles or virions are released from the infected cells by budding and are capable of infecting other permissive cells.

The infectious cycle of the adenovirus takes place in 2 steps:

- the early phase which precedes initiation of the replication of the adenoviral genome, and which permits production of the regulatory proteins participating in the replication and transcription of the viral DNA, and
- the late phase which leads to the synthesis of the structural proteins.

In general terms, the adenoviral genome consists of a double-stranded linear DNA molecule approximately 36 kb in length which contains the sequences coding for more than 30 proteins. At each of its ends, a short inverted sequence of 100 to 150 nucleotides, depending on the serotypes, designated ITR (inverted terminal repeat), is present. ITRs are involved in the replication of the adenoviral genome and also take part in the encapsidation. However, the actual encapsidation region of approximately 300 nucleotides is located at the 5' end of the genome immediately after the 5' ITR.

The early genes are distributed in 4 regions which are dispersed in the adenoviral genome, designated E1 to E4 (E denoting "early"). The early regions comprise at least six transcription units which possess their own promoters. The expression of the early genes is itself regulated, some genes being expressed before others. Three regions, E1, E2 and E4, respectively, are essential to the viral replication. Thus, if an adenovirus is defective for one of these functions, that is to say if it cannot produce at least one protein encoded by one of these regions, this protein will have to be supplied to it *in trans*.

The E1 early region is located at the 5' end of the adenoviral genome, and contains 2 viral transcription units, E1A and E1B, respectively. This region codes for proteins which participate very early in the viral cycle and are essential to the expression of almost all the other genes of the adenovirus. In particular, the E1A transcription unit codes for a protein which trans-activates the transcription of the other viral genes, inducing transcription from the

promoters of the E1B, E2A, E2B and E4 regions.

The products of the E2 region, which also comprises two transcription units E2A and E2B, are directly involved in the replication of the viral DNA. This region governs, in particular, the synthesis of a 72 kDa protein which displays a strong affinity for single-stranded DNA, and of a DNA polymerase.

The E3 region is not essential to the replication of the virus. It codes for at least six proteins which appear to be responsible for inhibition of the host's immune response with respect to an adenovirus infection. In particular, the gp19kDa glycoprotein appears to prevent the CTL response which is responsible for the cytolysis of infected cells by the host's cytotoxic T cells.

The E4 region is located at the 3' end of the adenoviral genome. It codes for many polypeptides which are involved in the expression of the late genes, the stability of late messengers (mRNAs), the transition from the early phase to the late phase and also the inhibition of cellular protein synthesis.

Once replication of the viral DNA has been initiated, transcription of the late genes begins. These occupy the majority of the adenoviral genome and partially overlap the transcription units of the early genes. However, they are transcribed from different promoters and according to an alternative mode of splicing, so that the same coding sequences are used for different purposes. Most of the late genes are transcribed from the major late promoter (MLP). This promoter permits the synthesis of a long primary transcript, which is then matured in the form of about twenty messenger RNAs (mRNAs) from which the capsid proteins of the virion are produced. Only the gene coding for structural protein IX of which the capsid is composed comprises its own promoter. This gene is located at the 5' end of the adenoviral genome and overlaps the E1B region at the 3' end of the E1 region. The protein IX transcription unit utilizes the same

transcription termination signal as the E1B transcription unit.

A number of adenoviruses are now well characterized genetically and biochemically. This is the case with human adenovirus type 5 (Ad5), the sequence of which is disclosed in the Genebank database under reference M73260. It has been possible to localize the different genes precisely on the adenoviral genome, as well as the encapsidation region (Hearing et al., 1987, J. Virol., *61*, 2555-2558). This region partly spans the regulatory region of the E1A transcription unit.

It emerges from the foregoing that adenoviruses possess advantageous features which make them vectors of choice for the transfer of genes of interest. Many recombinant adenoviruses are described in the literature (Rosenfeld et al., 1991, Science, *252*, 431-434; Rosenfeld et al., 1992, Cell, *68*, 142-155). Generally speaking, they are derived from Ad5, and are defective for the E1 function so as to avoid their dissemination in the environment and the host organism. In addition, the non-essential E3 region can also be deleted. Exogenous sequences are integrated in place of the E1 or E3 region.

Thus, these defective adenoviruses can be propagated only in a cell line complementing *in trans* the E1 function which is essential to viral replication. At present, the only complementation line which is usable is the embryonic kidney line 293 (Graham et al., 1977, J. Gen. Virol., *36*, 59-72), which results from the integration in its chromosomes of a fragment of the Ad5 genome comprising, in particular, the 5' end of the viral genome; so that line 293 complements adenoviruses which are defective for the E1 function. 293 cells contain sequences which are also found in the defective recombinant adenovirus, such as the 5' ITR, the encapsidation region and the portion 3' of the E1B region.

The feasibility of gene transfer using adenoviruses is now established. However, the question of

their safety has not yet been settled. In effect, they are capable of transforming some cell lines in culture, which reflects the potentially oncogenic power of some of the expression products of the adenoviral genome, essentially of the E1 and probably E4 regions. Furthermore, the probability of genetic recombination between a defective adenovirus of the prior art, in particular a recombinant adenovirus, and either a natural wild-type adenovirus (originating from an accidental contamination or from an opportunistic infection of a host organism) or an adenoviral genome fragment integrated in the complementation line 293, is not insignificant. In effect, one recombination event is enough to restore the E1 function and generate a non-defective recombinant adenovirus capable of being disseminated in the environment. It is also possible to envisage the situation where a natural wild-type adenovirus coinfecting the same cell as a defective adenovirus might complement the latter for the E1 function, causing a codissemination of the two viruses. Lastly, some types of eukaryotic cells produce proteins displaying an E1A-like activity, which are also capable of partially complementing the defective adenoviruses which infect them.

It is hence desirable to have at one's disposal efficacious adenoviruses affording a minimum of risk, with a view to their use in gene therapy for correcting *in vivo* serious genetic defects and treating certain disorders for which no effective therapeutic approaches are available. The success of gene therapy applied to man is dependent upon their being obtained.

Furthermore, doubts exist regarding the origin of line 293. These doubts can be liable to undermine the acceptability of products intended for use which are derived therefrom, for example a recombinant adenovirus. It would be useful to have at one's disposal complementation lines whose origin and history are precisely known.

There have now been found (1) novel defective

adenoviruses from which certain specific regions of the adenoviral genome have been deleted, and which are better suited to the transfer of an exogenous nucleotide sequence *in vivo*, and (2) novel, characterized complementation lines which are acceptable from a pharmaceutical standpoint and which hence afford all the safety features required for the production of products intended for human use.

The value of these novel adenoviruses is that they display an increased cloning capacity permitting the insertion of one or more large genes of interest, and afford maximal safety of use. These deleterious mutations render these adenoviruses incapable of autonomous replication and of cell transformation without impairing their capacity to transfer and express a gene of interest.

Thus, the present invention relates to an adenovirus which is defective for replication and which is selected from:

(a) an adenovirus which is derived from a natural adenovirus in that it lacks:

(i) at least the coding sequences of the E1B region, and/or

(ii) at least a part of the E4 region;

(b) an adenovirus which is derived from a natural adenovirus other than a human type 5 adenovirus in that it lacks a part of the encapsidation region.

The present invention also relates to an adenovirus which is defective for replication and which is selected from:

(a) an adenovirus which is derived from a natural adenovirus in that it lacks:

(i) at least the coding sequences of the E1B region, and/or

(ii) at least a part of the E4 region;

(b) an adenovirus which is derived from a human type 5 adenovirus in that it lacks:

(i) a part of the encapsidation region extending from nucleotide 270 to nucleotide 346 of the sequence as disclosed in the Genebank database under reference M73260, or

(ii) a part of the encapsidation region extending from nucleotide 184 to nucleotide 273 of the sequence as disclosed in the Genebank database under reference M73260.

Within the meaning of the present invention, the expression "defective adenovirus" refers to an adenovirus which is incapable of autonomous replication in a host cell but which is nevertheless infectious, that is to say has the ability to deliver its genome into a cell. An adenovirus according to the invention preferably lacks a part of the E1A region (other than the encapsidation region), like the defective adenoviruses of the prior art, and comprises a deletion in the encapsidation region and/or at least the coding sequences of the E1B region and/or all or part of the E4 region.

Within the meaning of the present invention, the expression "deletion" refers to the removal of at least one nucleotide in the targeted region.

A deletion of the E1B region will make it possible to reduce or remove the sequences which are shared in common between a defective adenovirus according to the invention and the adenoviral genome fragment which is integrated into a complementation

line, for example the line 293. Furthermore, this deletion eliminates, from the genome of an adenovirus according to the invention, sequences whose expression products are potentially oncogenic, at least in conjunction with the expression products of the E1A region. According to a preferred embodiment, the deletion of the E1B region affects the promoter and the sequences coding for the expression products of the E1B region and does not include all of the transcription termination signal.

A deletion of all or part of the E4 region will make it possible, at the same time, to increase the possibilities of cloning genes of interest and, as before, of reducing or removing sequences which encode potentially oncogenic products.

A deletion of a part of the encapsidation region will make it possible, in particular, to reduce the probability of a defective adenovirus according to the invention being disseminated in an uncontrolled manner when this virus is in the presence of a wild-type adenovirus. Such a deletion makes it possible to affect the encapsidation functions of an adenovirus according to the invention such that even if the defective function of this adenovirus were to be complemented by the wild-type adenovirus, its genome would not be able to be encapsidated efficiently as compared with the genome of the competing wild-type adenovirus. The deletions in the encapsidation region will be selected according to 2 criteria: a reduced ability to be encapsidated but, at the same time, a residual efficiency which is compatible with industrial production. A defective adenovirus according to the invention will exhibit an encapsidation efficiency which is reduced by a factor of from 2 to 50, advantageously of from 3 to 20, and preferably of from 5 to 10, as compared with a wild-type adenovirus.

A defective adenovirus according to the invention is advantageously selected from:

- an adenovirus which contains only one of the 3 abovementioned deletions, namely a defective adenovirus from which the E1B region, or all or part of the E4 region, or a part of the encapsidation region, has been deleted;

- an adenovirus which contains two deletions in any combination; and

- an adenovirus which contains all the deletions.

According to one advantageous embodiment, a defective adenovirus according to the invention is derived from a human type 5 adenovirus and contains:

(i) the deletion of at least the coding sequences of the E1B region, with said deletion terminating at nucleotide 4047 of the sequence as disclosed in the Genebank database under reference M73260; and/or

(ii) the deletion of all or part of the E4 region, said E4 region being located between nucleotides 32800 and 35826 of the sequence disclosed in the Genebank database under reference M73260; and/or

(iii) the deletion of a part of the encapsidation region:

- extending from nucleotide 270 to nucleotide 346 of the sequence as disclosed in the Genebank database under reference M73260, or

- extending from nucleotide 184 to nucleotide 273 of the sequence as disclosed in the Genebank database under reference M73260.

As pointed out previously, an adenovirus according to the invention is preferably defective for the E1A function and therefore lacks at least a part of the E1A region.

Furthermore, an adenovirus according to the invention can lack all or part of the E3 region. This deletion can be a deletion of 1 bp which destroys the reading frame of a gene, an intermediate deletion of one or more genes of the E3 region, or a deletion of the whole of the E3 region.

According to a particularly advantageous embodiment, only a part of the E3 region, and preferably the part which does not comprise the sequences coding for the gp19kDa protein, is deleted from an adenovirus according to the invention. The presence of the sequence coding for the gp19kDa protein in the genome of a defective adenovirus according to the invention will enable the infected cells to escape the immunosurveillance of the host; this is an important criterion when the therapeutic protocol necessitates several repeated administrations. Preference will be given to choosing to place the sequences coding for gp19kDa under the control of appropriate elements which enable gp19kDa to be expressed; namely the elements which are required for transcribing said sequences into mRNA and for translating the latter into protein. These elements comprise, in particular, a promoter. Such promoters are well known to the skilled person and are inserted upstream of said coding sequence using conventional techniques of genetic manipulation. Preferably, the selected promoter will be a constitutive promoter which cannot be activated by one of the expression products of the E1A region. Promoters which may be mentioned by way of example are the promoter of the HMG (hydroxymethylglutaryl coenzyme A reductase) gene, the SV40 (simian virus 40) virus early promoter, the RSV (Rous sarcoma virus) LTR (long terminal repeat) or the

promoter of a PGK (phosphoglycerate kinase) gene from a higher eukaryote.

Furthermore, a defective adenovirus according to the invention can additionally lack all or part of the E2 region.

According to a particularly preferred embodiment, an adenovirus according to the invention can lack all the genes coding for the early and late proteins of an adenovirus, the sole constraint being the presence, in the linear DNA molecule, of the 5' and 3' ITRs and of all or part of the encapsidation region. The genome of such a defective adenovirus according to the invention only comprises the minimum of viral sequence, in order to limit the risks of recombination and the risks of oncogenicity and to have maximum cloning capacity. Such a defective adenovirus will then be referred to as a "minimum" defective adenovirus. It will then be possible to insert up to 30 kb of exogenous nucleotide sequence into such an adenovirus.

In the context of the present invention, a defective adenovirus according to the invention has as its objective the transfer of an exogenous nucleotide sequence to a host cell and its expression therein. This is why a defective adenovirus according to the invention advantageously comprises an exogenous nucleotide sequence. "Exogenous nucleotide sequence" is understood to mean a nucleic acid which comprises coding sequences and regulatory sequences permitting the expression of said coding sequences, and in which the coding sequences are sequences which are not normally present in the genome of an adenovirus. The regulatory sequences can be of any origin. The exogenous nucleotide sequence is introduced into a defective adenovirus according to the invention by standard techniques of genetic engineering.

An exogenous nucleotide sequence can consist of one or more gene(s) of interest, and preferably of therapeutic interest. In the context of the present invention, a gene of interest can code either for an antisense RNA, or for an mRNA which will then be trans-

lated into a protein of interest. A gene of interest can be of genomic type, of complementary DNA (cDNA) type or of mixed or minigene type, in which at least one intron is deleted. It can code for a mature protein, a precursor of a mature protein, in particular a precursor intended to be secreted and accordingly comprising a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origins, or a mutant of a natural protein displaying improved or modified biological properties. Such a mutant may be obtained by mutation, deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the natural protein.

A gene of interest may be placed under the control of elements suitable for its expression. "Elements suitable for their expression" are understood to mean the set of elements needed for its transcription into RNA (antisense RNA or mRNA) and for the translation of an mRNA into protein. Among the elements needed for transcription, the promoter assumes special importance. In a general manner, a promoter will be selected which functions in the cells which it is sought to infect with a defective adenovirus according to the invention. The retained promoter can be a constitutive promoter or a regulable promoter. It can be isolated from a gene of a eukaryotic cell or a virus, and even an adenovirus, such as, for example, the MLP promoter. It can be the natural promoter of the gene of interest in question. It may be modified so as to contain regulatory sequences. As examples, the gene(s) of interest making up the exogenous nucleotide sequence are placed under the control of the promoter of the immunoglobulin genes when it is desired to target the transfer of the exogenous nucleotide sequence to lymphocytic cells. There may also be mentioned the TK-HSV-1 (herpesvirus, type 1 thymidine kinase) gene promoter, which is a constitutive promoter, permitting expression in a large number of cell types.

Among genes of interest which are usable in the context of the present invention, there may be

mentioned:

- the genes coding for cytokines such as interferon alpha, interferon gamma, interleukins;
- the genes coding for membrane receptors such as the receptors recognized by pathogenic organisms (viruses, bacteria or parasites), preferably by the HIV virus (human immunodeficiency virus);
- the genes coding for coagulation factors such as factor VIII and factor IX;
- the gene coding for dystrophin;
- the gene coding for insulin;
- the genes coding for proteins participating directly or indirectly in cellular ion channels, such as the CFTR (cystic fibrosis transmembrane conductance regulator) protein;
- the genes coding for antisense RNAs or proteins capable of inhibiting the activity of a protein produced by a pathogenic gene, present in the genome of a pathogenic organism, or by a cellular gene, the expression of which is deregulated, for example an oncogene;
- the genes coding for a protein inhibiting an enzyme activity, such as $á_1$-antitripsin or a viral protease inhibitor;
- the genes coding for variants of pathogenic proteins which have been mutated so as to impair their biological function, such as, for example, trans-dominant variants of the TAT protein of the HIV virus which are capable of competing with the natural protein for binding to the target sequence, thereby preventing the activation of HIV;
- the genes coding for antigenic epitopes in order to increase the host cell's immunity;
- the genes coding for major histocompatibility complex classes I and II proteins, as well as the genes coding for the proteins which are inducers of these genes;

- the genes coding for cellular enzymes or those produced by pathogenic organisms; and
- suicide genes. The TK-HSV-1 suicide gene may be mentioned more especially. The viral TK enzyme displays markedly greater affinity compared to the cellular TK enzyme for certain nucleoside analogues (such as acyclovir or gancyclovir). It converts them to monophosphated molecules, which can themselves be converted by the cellular enzymes to nucleotide precursors, which are toxic. These nucleotide analogues can be incorporated in DNA molecules undergoing synthesis, hence chiefly in the DNA of cells in a state of replication. This incorporation enables dividing cells such as cancer cells to be destroyed specifically.

This list is not restrictive, and other genes may be used in the context of the present invention.

Moreover, according to another embodiment of the invention, a defective adenovirus according to the invention can, in addition, comprise a non-therapeutic gene coding for a protein which trans-activates transcription. Naturally, the gene(s) of the E1A region coding for a trans-activating protein, the expression of which would run the risk of rendering the adenovirus non-defective, will be avoided. The gene coding for the *Saccharomyces cerevisiae* Gal4 protein will preferably be chosen. The expression of such a gene in an adenovirus according to the invention will enable it to be propagated in a new complementation line such as the one described below. Such a line is more sophisticated, and enables possible problems of toxicity due to the continuous production of adenoviral complementation proteins to be alleviated. The gene coding for a protein which trans-activates transcription may be placed, if necessary, under the control of elements suitable for its expression; for example those which permit the expression of a gene of interest.

A defective adenovirus according to the present

invention is derived from a natural adenovirus, advantageously a canine, avian or human adenovirus, preferably a human type 2, 3, 4, 5 or 7 adenovirus, and very preferably a human type 5 adenovirus.

The invention also relates to a eukaryotic cell comprising a defective adenovirus according to the invention. Said cell is advantageously a mammalian cell, and preferably a human cell.

A defective adenovirus according to the invention may especially be prepared by passage in a complementation line providing *in trans* the functions for which an adenovirus according to the invention is defective.

Thus, the present invention also relates to a complementation line formed by a cell line containing a complementation element, comprising at least a portion of the E1 region of a natural adenovirus and lacking the 5' ITR sequence; the complementation element being either integrated in the genome or inserted into a vector.

In the context of the present invention, the term "complementation line" refers to a cell line capable of providing *in trans* the function(s) for which an adenovirus is defective; in other words, of producing all the proteins needed for forming a viral particle, which elements cannot be produced by a defective adenovirus itself. A complementation line according to the invention may be derived either from an immortalized cell line capable of dividing indefinitely, or from a primary line. In accordance with the objectives pursued by the present invention, a complementation line according to the invention is useful for the propagation of any defective adenovirus, and especially a defective adenovirus according to the invention. Thus, when the broad term "defective adenovirus" is used, it should be understood to also include a defective adenovirus according to the invention.

A complementation line according to the invention contains a complementation element.

"Complementation element" is understood as meaning a nucleic acid which comprises one or more gene(s), in particular isolated from a natural adenovirus, whose expression will make it possible to supply in trans the corresponding adenoviral protein(s) and to complement the function(s) for which an adenovirus is defective. The complementation element can be inserted into a vector, for example of the plasmid or viral type, or, preferably, integrated into the genome of a complementation line according to the invention. The methods for introducing a vector or a nucleic acid into a cell line, and of integrating said nucleic acid in the genome of a cell, constitute conventional techniques well known to a person skilled in the art. The latter also knows the different vectors which are usable for such purposes. The complementation element may be introduced into a complementation line according to the invention, beforehand or concomitantly with a defective adenovirus.

A complementation line according to the invention is intended to complement *in trans* an adenovirus which is defective for the E1 function, like, for example, the defective adenoviruses of the prior art, or a defective adenovirus according to the invention, from which the E1B region has been deleted. Such a line has the advantage of decreasing the risks of recombination since, contrary to the conventional line 293, the complementation element which it contains lacks the 5' ITR.

Moreover, according to another embodiment of the invention, a complementation line according to the invention can include a complementation line lacking, also, the promoter of the E1A region, the E1A promoter being replaced with an appropriate non-adenoviral promoter. In a general manner, a promoter will be chosen which functions in the cell line from which said complementation line is derived. The appropriate promoter can be isolated from any viral, apart from adenoviral, gene or from a eukaryotic gene. The

promoter in question can be a constitutive promoter. As examples, the SV40 virus, TK-HSV-1 gene and murine PGK gene promoters may be mentioned.

Alternatively, the promoter selected may be regulable and advantageously inducible by a protein which trans-activates transcription. It can be a promoter isolated from a naturally inducible gene or any promoter modified by the addition of activating sequences or UAS (upstream activating sequence) responding to said trans-activating protein. More especially, a promoter will be used which is inducible by the *Saccharomyces cerevisiae* Gal4 protein, and preferably a so-called "minimum" promoter of the TK-HSV-1 gene containing only the transcription initiation sequences, upstream of which at least one UAS of the *Saccharomyces cerevisiae* Gal10 gene has been inserted. The UASs may be synthesized chemically or isolated from the Gal10 gene according to standard techniques of genetic engineering. Thus, such a promoter will be activated, and will induce the expression of the genes encoded by the E1A region placed under its control, only in the presence of the Gal4 protein. Moreover, the E1B promoter can itself be induced by the trans-activating protein encoded by the E1A region. This particular embodiment of the invention avoids the constitutive (possibly toxic) production of the proteins complementing the entire E1 function. Their production is triggered only in the presence, for example, of a defective adenovirus according to the invention expressing the Gal4 protein. Such a line may also be used to prepare any adenovirus which is defective for the E1 function, on condition, however, of providing the Gal4 protein *in trans*. The means of providing a protein *in trans* are known to a person skilled in the art.

According to an advantageous embodiment, a complementation line according to the invention contains a complementation element which comprises at least a part of the E1 region of a human type 5 adenovirus, which part is selected from:

(i) the region, or a homologous region, proceeding from nucleotide 100 and terminating at nucleotide 5297 of the sequence as disclosed in the Genebank database under reference M73260, and

(ii) the region, or a homologous region, proceeding from nucleotide 100 and terminating at nucleotide 4034 of the sequence as disclosed in the Genebank database under reference M73260, and

(iii) the region, or a homologous region, proceeding from nucleotide 505 and terminating at nucleotide 4034 of the sequence as disclosed in the Genebank database under reference M73260 and placed under the control of the elements which are required for expression of said region.

Advantageously, a complementation element comprising the region proceeding from nucleotide 100 and terminating at nucleotide 4034 of a human type 5 adenovirus will additionally contain a transcription termination signal, like, for example, the polyadenylation signal of the SV40 (simian virus 40) virus or of the rabbit β-globin gene.

Furthermore, the region proceeding from nucleotide 505 and terminating at nucleotide 4034 of a human type 5 adenovirus corresponds to all the coding sequences of the E1 region but does not comprise either the promoter sequences of the E1A region or the transcription termination signal of the E1B region. Thus, a complementation element which contains this region (505-4034) will additionally comprise the appropriate elements enabling it to be expressed. In particular, an appropriate non-adenoviral promoter, as previously described, will be inserted at 5′ of said

region, and a transcription termination signal, for example that of the rabbit β-globin gene, will be inserted at 3'. A complementation line which contains such a complementation element will constitute a "clean" complementation line, which lacks the majority of the sequences common to a defective adenovirus.

A complementation line according to the invention can contain a complementation element which additionally comprises at least a part of the E4 region of a natural adenovirus, preferably at least a part of the E4 region of a human type 5 adenovirus (said E4 region proceeding from nucleotide 32800 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260).

Within the context of the invention, the part of the E4 region can be placed, if necessary, under the control of appropriate elements which enable it to be expressed. Alternatively, and preferably, the part of the E4 region included in the complementation element comprises its own promoter, which promoter can be induced by the transcription trans-activating protein encoded by the E1A region.

Such a complementation line is intended for preparing an adenovirus which is defective for the E1 and E4 functions, such as, for example, a defective adenovirus according to the invention from which the E1B region and all or part of the E4 region have been deleted. Furthermore, within the context of the particular embodiment of the invention according to which the promoter of the E1A region is replaced with a promoter which can be induced by the Gal4 protein, such a complementation line will only produce the proteins for complementing the E1 and E4 functions in the presence of a defective adenovirus which expresses the Gal4 protein, thereby avoiding the problems of toxicity which are linked to the continuous production of adenoviral proteins.

A complementation line according to the invention can contain a complementation element which

additionally comprises a part of the E2 region from a natural adenovirus. Such a line will make it possible to complement, *in trans*, all the functions which are essential for the replication of an adenovirus and is therefore particularly suited to propagating an adenovirus which is defective for the E1, E2 and E4 functions, in particular a defective adenovirus according to the invention such as a minimum adenovirus.

Furthermore, a complementation line according to the invention contains a complementation element which comprises the whole of the genome of a natural adenovirus apart from the encapsidation region and the 5' ITR region of said adenovirus, very particularly preferably the part of the genome of a human type 5 adenovirus proceeding from nucleotide 505 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260.

For the purposes of the present invention, the part of the genome of a human type 5 adenovirus proceeding from nucleotide 505 and terminating at nucleotide 35826 can be placed under the control of an appropriate non-adenoviral promoter. Such a promoter has been previously described and preference will also be given to a promoter which can be induced by the *Saccharomyces cerevisiae* Gal4 protein.

According to a preferred embodiment, a complementation line according to the invention can contain a complementation element comprising, in addition, a gene coding for a selectable marker permitting the detection and isolation of the cells containing it. In the context of the present invention, this can be any gene coding for a selectable marker, such genes being generally known to a person skilled in the art, advantageously a gene for resistance to an antibiotic, and preferably the gene coding for puromycin acetyltransferase (pac gene) conferring resistance to puromycin.

In the context of the present invention, the gene coding for a selectable marker may also be placed under the control of suitable elements permitting its expression. Of course, these elements comprise any promoter. Said gene may be placed under the control of a constitutive promoter, especially the SV40 virus early promoter. However, a promoter which is inducible by the trans-activating protein encoded by the E1A region will be preferred, especially the E2A adenoviral promoter. Such a combination will induce a selection pressure to maintain a complementation element including and expressing the E1A region in a complementation line according to the invention. For the purposes of the present invention, the promoter selected may be modified by deletion, mutation, substitution and/or addition of nucleotides.

According to an absolutely preferred embodiment, a complementation line according to the invention is formed by a cell line which is acceptable from a pharmaceutical standpoint. "Cell line which is acceptable from a pharmaceutical standpoint" is understood to mean a cell line which is characterized (whose origin and history are known) and/or which has already been used for the large-scale production of products intended for human use (assembly of batches for advanced clinical trials or of batches intended for sale). In this connection, there may be mentioned the Vero African green monkey kidney and BHK golden or Syrian hamster kidney lines, the A549 human line derived from a lung carcinoma, and the MRC5 human pulmonary, W138 human pulmonary and CHO Chinese hamster ovary lines.

The invention also relates to a method for preparing a defective adenovirus according to the invention, according to which:

- said defective adenovirus is introduced into a complementation line according to the invention,
- said complementation line is cultured according to suitable conditions for permitting the

production of said defective adenoviruses, and

- said defective adenoviruses are recovered in the culture supernatant of said line.

Within the context of the invention, an adenovirus according to the invention can alternatively be prepared using the conventional techniques known to the skilled person (Graham and Prevec, in Methods in Molecular Biology, Vol. 7, 109-128, Ed: E.J. Murey, The Human Press Inc.).

The subject of the invention is also the therapeutic or prophylactic use of a defective adenovirus according to the invention, a eukaryotic cell according to the invention or a complementation line according to the invention.

Lastly, the present invention relates to a pharmaceutical composition comprising as therapeutic or prophylactic agent a defective adenovirus according to the invention, a eukaryotic cell according to the invention or a complementation line according to the invention, in combination with a vehicle which is acceptable from a pharmaceutical standpoint.

The composition according to the invention is intended especially for the preventive or curative treatment of disorders such as:

- genetic disorders such as, for example, hemophilia, cystic fibrosis or Duchêne's and Becker type myopathies,
- cancers such as, for example, those induced by oncogenes or viruses,
- retroviral diseases such as, for example, AIDS (acquired immunodeficiency syndrome resulting from HIV infection), and
- recurrent viral diseases such as, for example, herpesvirus-induced infections.

A pharmaceutical composition according to the invention may be manufactured in a conventional manner. In particular, a therapeutically effective amount of a therapeutic or prophylactic agent is combined with a vehicle such as a diluent. A composition according to

the invention may be administered by aerosol or via any conventional route in use in the field of the art, especially via the oral, subcutaneous, intramuscular, intravenous, intraperitoneal, intrapulmonary or intratracheal route. The administration may take place in a single dose or a dose repeated one or more times after a certain time interval. The appropriate administration route and dosage vary in accordance with various parameters, for example with the individual being treated or the disorder to be treated, or alternatively with the gene(s) of interest to be transferred. Generally speaking, a pharmaceutical composition according to the invention comprises a dose of adenovirus according to the invention of between 10^4 and 10^{14}, advantageously 10^5 and 10^{13} and preferably 10^6 and 10^{11}. A pharmaceutical composition can comprise, in addition, an adjuvant which is acceptable from a pharmaceutical standpoint.

The invention also encompasses a method of treatment, according to which a therapeutically effective amount of a defective adenovirus according to the invention, a eukaryotic cell according to the invention or a complementation line according to the invention is administered to a patient requiring such treatment.

The present invention is described more completely by means of the examples which follow.

EXAMPLES

The examples which follow illustrate only one embodiment of the present invention.

The constructions described below are carried out according to the general techniques of genetic engineering and molecular cloning detailed in Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). The collective steps of cloning employing bacterial plasmids are carried out by passage in *Escherichia coli (E. coli)* strain 5K, whereas those employing vectors derived from phage M13 are carried out by passage in *E. coli* NM522.

As regards the steps of PCR amplification, the protocol as described in PCR Protocols - A guide to methods and applications (1990, edited by Innis, Gelfand, Sninsky and White, Academic Press Inc.) is applied.

The fragments inserted into the different constructions described below are indicated precisely according to their position in the nucleotide sequence of:

- the Ad5 genome, as disclosed in the Genebank database under reference M73260,
- the adenovirus type 2 (Ad2) genome, as disclosed in the Genebank database under reference J01949,
- the SV40 virus genome, as disclosed in the Genebank database under reference J02400.

EXAMPLE 1: Generation of an "attenuated" virus comprising a deletion of a portion of the encapsidation region

1. *Construction of an "attenuated" virus comprising a deletion from nucleotide 184 to nucleotide 273 of the encapsidation region*

a. *Generation of the deletion*

A vector comprising the following is constructed:

- the ITR situated at the 5' end of the Ad5 genome (from nucleotide 1 to nucleotide 103),
- the Ad5 encapsidation region lying between nucleotides 104 and 458, in which the portion ranging from nucleotide 184 to nucleotide 273 is deleted and the thymine (T) at position 176 is modified to a cytosine (C) in order to create an *Aat*II restriction site,
- a cassette for the expression of a gene of interest comprising, from 5' to 3', the Ad2 MLP (nucleotides 5779 to 6038), the *Kpn*I-*Xba*I-*Hind*III and *Bam*HI restriction sites, the human cDNA coding for the CFTR protein (the amino acid composition corresponds to the sequence published by Riordan et al., 1989, Science, *245*, 1066-1073; with the

exception of a valine in place of the methionine at position 470), the *Pst*I, *Xho*I and *Sal*I sites and lastly the SV40 virus transcription termination signal (nucleotides 2665 to 2538),

- the fragment of the Ad5 genome extending from nucleotide 3329 to nucleotide 6241.

In a first stage, the *Eco*RI *Sma*I fragment isolated from pMLP11 is cloned between the *Eco*RI and *Eco*RV sites of the vector M13TG131 (Kieny et al., 1983, Gene, *26*, 91). This construction originates from pMLP10 (Levrero et al., 1991, Gene, *101*, 195-202), and differs from the parent vector by the introduction of an *Sma*I site at the *Hind*III site. The vector M13TG6501 is obtained. The latter is subjected to a directed mutagenesis in order to delete the sequences lying between nucleotides 184 and 273 of the encapsidation region. The directed mutagenesis is carried out according to the standard techniques, and employs the oligonucleotide OTG4174 listed under sequence identifier No. 1 (SEQ ID NO: 1). The mutated vector is designated M13TG6502. The encapsidation region thus deleted is reintroduced in the form of an *Eco*RI-*Bgl*II fragment, the *Bgl*II site being rendered blunt by treatment with Klenow DNA polymerase, into the vector pMLP11 digested with *Eco*RI and *Sma*I.

The vector obtained, pTG6500, is partially digested with *Pst*I, treated with phage T4 DNA polymerase and then digested with *Pvu*I. The *Pvu*I-*Hpa*I fragment isolated from pIX is inserted into this vector. This fragment contains the SV40 virus transcription termination signal and the adenoviral fragment of nucleotide 3329 to nucleotide 6241. The vector pTG6505 thus generated is partially digested with *Sph*I, treated with phage T4 DNA polymerase and religated, the purpose of this being to destroy the *Sph*I site located at the 5' end of the polylinker. This results in pTG6511, into which, after *Bam*HI digestion and treatment with Klenow DNA polymerase, human CFTR cDNA is cloned in the form of a blunt-ended fragment generated by *Xho*I-*Ava*I digestion

and treatment with Klenow DNA polymerase. pTG6525 is obtained.

b. Generation of a defective and attenuated recombinant adenovirus

The defective recombinant adenoviruses are generated by homologous recombination using standard techniques. More specifically, the plasmid pTG6525 is linearized with *Cla*I and cotransfected into 293 cells together with an Ad-dl324 DNA (Thimmappaya et al., 1982, Cell, *31*, 543-551) which is also digested with ClaI. The recombinant adenoviral DNA is replicated and encapsidated in infectious virions, forming plaques which are screened using standard procedures. The individual plaques which are selected are amplified in 293 cells. Viral stocks are prepared and their titres are determined using conventional techniques.

2. Construction of an "attenuated" virus comprising a deletion from nucleotide 270 to nucleotide 346 of the encapsidation region

First of all, a plasmid is constructed which corresponds to pTG6525 but in which the sequences contained between nucleotides 270 and 346 have been deleted from the encapsidation region.

The vector M13TG6501 is subjected to a directed mutagenesis employing the oligonucleotide OTG4173 reported in SEQ ID NO: 2. The mutated fragment is then isolated after digesting with *Bgl*II, treating with Klenow DNA polymerase and digesting with *Eco*RI. It is introduced between the *Eco*RI and *Sma*I sites of the plasmid pMLP11 in order to generate the vector pTG6501. The latter is digested with *Sph*I, and treated with phage T4 DNA polymerase and digested with *Pvu*I. A *Pvu*I-*Kpn*I fragment, the *Kpn*I site having been rendered blunt, is cloned into the pTG6501 which has been treated in this way. This fragment is isolated from pTG6525 and contains the human CFTR cDNA.

The attenuated viruses are generated by homologous recombination according to the protocol

described above.

EXAMPLE 2: Construction of a defective adenovirus from which the E1B region has been deleted

Such an adenovirus is generated from a plasmid vector comprising, from 5' to 3':

- the Ad5 5' ITR (from nucleotide 1 to nucleotide 103),
- the Ad5 encapsidation region (from nucleotide 104 to nucleotide 458),
- an exogenous nucleotide sequence containing an expression cassette which comprises the following elements:
  - the Ad2 MLP (nucleotides 5779 to 6038), followed by three tripartite leaders, also of Ad2 (nucleotides 6039-6079; nucleotides 7101-7175; nucleotides 9637-9712); these leaders are included in order to increase the efficiency of translation of the sequences inserted downstream,
  - a polylinker comprising, from 5' to 3', the *Xba*I *Hind*III, *Bam*HI and *Not*I restriction sites which are usable for the cloning of a gene of interest,
  - a gene of interest,
  - the transcription termination signal isolated from the SV40 virus (nucleotides 2543 to 2618),
- the portion of the Ad5 adenoviral genome ranging from nucleotide 4047 to nucleotide 6241.

Such an adenovirus can be constructed in the same manner.

The fragment of the adenoviral genome, from nucleotide 4047 to nucleotide 4614, is amplified by PCR using the vector pBRE1. The vector pBRE1 is derived by inserting a fragment, containing the 5' end of Ad5 up to the *Xho*I site in position 5778, between the *Hind*III site, which has been rendered blunt, and the *Sal*I site of pBR322 (Bolivar et al., 1977, Gene, 2, 95-113). The

PCR reaction uses the sense primer OTG5021 (reported in SEQ ID NO:3), which, at its 5' end, comprises a *Bam*HI site which is intended to facilitate the subsequent cloning steps, and the antisense primer OTG5157, which is described in SEQ ID NO:4. The fragment which is generated is treated with Klenow DNA polymerase in order to generate blunt ends and cloned into the *Sma*I site of M13mp18 (Gibco BRL), giving rise to M13TG6517. The sequence of the PCR-generated fragment is verified using the standard enzymic method (Sanger et al., *1977*, Proc. Natl. Acad. Sci. USA, *74*, 5463).

Separately, the *Pvu*I-*Sma*I fragment is isolated from pMLP11. It is cloned between the *Pvu*I and *Kpn*I sites of pTG6511 (described in Example 1), the *Kpn*I site having been rendered blunt by treatment with phage T4 DNA polymerase according to standard methods. The vector pTG6547 is thereby generated.

pTG6547 is digested with the enzymes *Sal*I and *Bst*XI and ligated to two fragments, on the one hand the purified *Bam*HI-*Bst*XI fragment of M13TG6517, and on the other hand the *Xho*I and *Bgl*II fragment of pTG6185. pTG6185 comprises, in particular, the SV40 virus transcription termination signal flanked by the *Xho*I and *Bgl*II restriction sites. However, any other plasmid containing the same termination sequence and appropriate restriction sites could be used. The vector pTG6555 is obtained.

The gene of interest is inserted into the polylinker intended for this purpose. For example, human CFTR cDNA, in the form of an *Sac*I-*Pst*I fragment with the *Pst*I site blunt, is inserted into the pTG6555 *Bam*HI site which has been rendered blunt. As mentioned previously, the amino acid composition of the CFTR protein corresponds to the sequence published by Riordan et al., (supra), with the exception of a valine in place of the methionine in position 470.

The defective adenoviruses from which the E1B region has been deleted and the recombinant adenoviruses are generated by homologous recombination

according to the protocol described previously.

EXAMPLE 3: Construction of a defective "minimum" virus

The genome of a minimum adenovirus is formed from a DNA molecule comprising:

- the Ad5 5' ITR (from nucleotide 1 to nucleotide 103);
- the Ad5 encapsidation region (from nucleotide 104 to nucleotide 458);
- an exogenous nucleotide sequence comprising:
  - a first gene of therapeutic interest, preferably placed under the control of its own promoter in order to obtain a regulation of expression which is as close as possible to the natural regulation,
  - a second gene of interest consisting of the TK-HSV-1 gene, and
  - optionally, nucleotide sequences of any kind, added for reasons of efficiency of encapsidation so that the total size of the genome to be encapsidated is between 30 and 36 kb;
- the sequences coding for the *Saccharomyces cerevisiae* Gal4 protein (Laughon and Gesteland, 1984, Mol. Cell. Biol., *4*, 260-267), placed under the control of a promoter which is functional in a higher eukaryotic cell; and
- The Ad5 3' ITR (from nucleotides 35833 to 35935).

Assembly of the different portions of the genome is carried out according to standard techniques of molecular biology. The production of infectious virions comprising such a genome is carried out as described above.

EXAMPLE 4: Formation of a complementation line of the E1 function

1. *Construction of a complementation element comprising the E1 region of Ad5*

a. Construction of pTG6533

This cell comprises a complementation element which contains:

- a cassette for the expression of the pac gene, which gene is placed under the control of the SV40 virus early promoter (nucleotides 5171 to 5243) and comprises at the 3' end the SV40 transcription termination signal (nucleotides 2543 to 2618). The pac gene used corresponds to a fragment ranging from nucleotide 252 to nucleotide 905 of the sequence disclosed by Lacalle et al. (1989, Gene, *79*, 375-380) and containing 4 mutations relative to the published sequence (C at position 305 replaced by A; C at position 367 replaced by T; insertion of a G at position 804; deletion of a G at position 820),
- a fragment of the Ad5 genome ranging from nucleotide 100 to nucleotide 5297. This fragment comprises the E1 region, equipped with its own promoter and its transcription termination signal, as well as a fraction of the E2 region.

The construction is carried out in several steps detailed below. The vector p polyIII-I* (Lathe et al., 1987, Gene, *57*, 193-201) is subjected to digestion with the enzymes *Acc*I and *Eco*RI. The *Eco*RI-*Cla*I fragment isolated from plasmid pTG6164 is cloned into the vector thus treated. The vector pTG6528 is obtained.

Plasmid pTG6164 originates from pLXSN (Miller D, 1989, Bio/Techniques, 7, 980) and comprises the pac gene placed under the control of the SV40 virus early promoter. Briefly, the *Hind*III-*Kpn*I fragment of pLXSN is introduced into M13TG131 (Kieny et al., supra) to produce M13TG4194. The *Nhe*I-*Kpn*I fragment of pMPSV H2 K IL2R (Takeda et al., 1988, Growth Factors, *1*, 59-66) is inserted into the latter, digested with *Nhe*I and *Kpn*I, to produce M13TG4196. The latter is digested with *Hind*III-*Kpn*I, and the purified fragment of pLXSN originating from a *Hind*III digestion and a partial *Kpn*I

digestion is cloned. pTG5192 is obtained. The latter is digested with *Hind*III and partially with *Nhe*I, and the *Hind*III-*Nhe*I fragment of pBabe Puro (Land et al., 1990, Nucleic Acids Res., *18*, 3587) is introduced, giving rise to pTG6164.

The vector pTG6528 is digested with *Pst*I, and the *Pst*I fragment isolated from pTG6185 (see Example 2) containing the SV40 transcription termination signal is introduced at this site. pTG6529 is obtained. The latter is subjected to *Eco*RI-*Hpa*I digestion and ligated to two fragments, on the one hand a fragment generated by PCR at the *Eco*RI and *Bsp*EI ends and on the other hand a purified *Bsp*EI-*Bcg*I fragment of pBRE1. pTG6531 is produced.

The PCR fragment is generated by gene amplification from pBRE1 with the aid of the primers OTG4564 and OTG4565 (listed under SEQ ID NO: 5 and 6). The amplified fragment is cut with the enzymes *Eco*RI and *Bsp*EI, and ligated as described in the preceding paragraph.

The vector pTG6531 comprises the 2 transcription units (that of the E1 region and that of the pac gene) in the same orientation. To avoid interference in respect of transcription, they are placed in a reverse orientation by cutting pTG6531 with *Bam*HI and religating. The clone pTG6533 corresponds to a clone displaying the desired, i.e. reverse orientation of the two units.

b. Construction of pTG6557, pTG6558, pTG6564 and pTG6565

The vectors pTG6557 and pTG6558 comprise a complementation element containing:

- a cassette for the expression of the pac gene (nucleotides 252 to 905 as before) under the control either of the native E2A promoter of Ad2 (nucleotides 27341 to 27030) in pTG6558, or of the E2A promoter of Ad2 from which has been

deleted the sequence lying between nucleotides 27163 and 27182, in pTG6557. Such a mutation enables the baseline level of the E2A promoter to be decreased without affecting the inducibility by the trans-activating protein encoded by E1A. Said expression cassette contains at 3' the SV40 virus transcription termination signal (nucleotides 2543 to 2618); and

- an expression cassette containing the portion of the Ad5 E1 region ranging from nucleotide 505 to nucleotide 4034. This portion of the adenoviral genome contains the whole of the sequences coding for the E1A region, the transcription termination signal of the E1A unit, the E1B promoter (inducible by the trans-activating protein encoded by E1A) and the coding sequences of the E1B region. It also includes the sequences coding for protein IX, which cover the E1B region. However, it lacks the promoter of the E1A region and the transcription termination signal of the E1B and IX transcription units. In order to permit the expression of the coding sequences carried by this portion of the adenoviral genome, the complementation element comprises the murine PGK gene promoter at the 5' end of the adenoviral fragment, and the transcription termination signal of the rabbit β-globin gene (nucleotides 1542 to 2064 of the sequence disclosed in the Genebank database under reference K03256) at the 3' end.

Optionally, nucleotide sequences of any kind, for example sequences isolated from pBR322, may be introduced between the cassettes for the expression of the pac gene and of the E1 region, in order to avoid possible interference with transcription. Such constructs correspond to pTG6564 which is derived from pTG6557, and pTG6565 which is derived from pTG6558.

The complementation element borne by one of

these 4 vectors has the advantage over the one borne by pTG6533 of containing only the sequences required for complementation, that is to say the coding sequences of the E1 region. The construction of these vectors is performed in several steps reported below.

First, the fragment of the Ad5 genome ranging from nucleotide 505 to nucleotide 826 is amplified by PCR from vector pBRE1 and using the primers OTG5013, which comprises at the 5' end a *Pst*I site which is useful for the subsequent cloning steps and which is described in SEQ ID NO: 7, and OTG4565 overlapping the *Bsp*EI site (SEQ ID NO: 6). The fragment generated by PCR is treated with Klenow DNA polymerase and then introduced into the *Sma*I site of M13mp18 (Gibco BRL), giving rise to M13TG6512. The sequence of the PCR fragment is verified.

The vector pTG6533 (see Example 4; comprising the E1 region of Ad5 from nucleotide 100 to nucleotide 5297) is digested with the enzymes *Eco*RI and *Bsp*EI. The vector thus treated is ligated with, on the one hand the *Pst*I-*Bsp*EI fragment isolated from M13TG6512, and on the other hand the *Eco*RI-*Pst*I fragment isolated from pKJ-1. The latter fragment comprises the portion of the murine PGK gene promoter lying between nucleotides -524 and -19, the sequence of which is reported in Adra et al. (1987, Gene, *60*, 65-74). This step which generates pTG6552, enables the murine PGK gene promoter to be inserted upstream of the E1 region of Ad5 beginning at nucleotide 505.

Separately, the *Xho*I-*Bam*HI fragment, of which the end generated by *Xho*I is rendered blunt following treatment with Klenow DNA polymerase, is purified from pBCMG Neo (Karasuyama et al., 1989, J. Exp. Med., *169*, 13-25). This fragment, which comprises the transcription termination signal of the rabbit â-globin gene, is introduced between the *Sma*I and *Bam*HI sites of the vector p polyII-Sfi/Not-14* (Lathe et al., 1987, Gene, *57*, 193-201). The vector pTG6551 which results is, for its part, digested with the enzymes *Sph*I and *Eco*RV in

order to insert into it a fragment of Ad5 genome ranging from nucleotide 3665 to nucleotide 4034. This fragment is generated by PCR according to the standard protocol. The procedure used employs the vector pBRE1 as template, and the primers OTG5015 (which overlaps the internal *Sph*I site at position 3665 and which is described in SEQ ID NO: 8) and OTG5014 (comprising at the 5' end a *Bgl*II site and reported in SEQ ID NO: 9).

The PCR fragment, treated with Klenow DNA polymerase, is cloned into the *Sma*I site of M13mp18, generating M13TG6516. After verification of its sequence, the PCR fragment is abstracted by *Bgl*II digestion, treatment with Klenow DNA polymerase and *Sph*I digestion. It is inserted between the *Sph*I and *Eco*RV sites of pTG6551. This results in pTG6554.

Separately, the vector pTG6529 (described in Example 4) is subjected to digestion with the enzymes *Hpa*I and *Hind*III. The 2.9-kb fragment containing the pac gene followed by the SV40 virus transcription termination signal is purified. This fragment is ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac (Boeuf et al., 1990, Oncogene, *5*, 691-699) which carries the Ad2 E2A promoter. The vector pTG6556 is obtained. Alternatively, it may be ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac Δ9170 (Zajchowski et al., EMBO J., *4*, 1293-1300), which carries the mutated E2A promoter of Ad2. In this case, pTG6550 is obtained.

pTG6556 is digested with the enzymes *Eco*RI and *Bam*HI. The *Eco*RI-*Sac*II fragment isolated from pTG6552 and the *Sac*II-*Bam*HI fragment isolated from pTG6554 are inserted between these sites. The vector pTG6558 is obtained. The same step carried out on pTG6550 generates pTG6557.

pTG6557 and pTG6558 are digested with *Eco*RV, a unique site located between the two expression cassettes (pac gene and E1 region). A 1.88-kb *Eco*RV-*Pvu*II fragment isolated from pBR322 (Bolivar et al., *supra*) is cloned into this site in order to increase the distance between the two promoters. pTG6564 and pTG6565, respectively,

are generated.

2. Generation of a complementation line of the E1 function

The vector pTG6533, pTG6557, pTG6558, pTG6564 or pTG6565 is transfected into a Vero cell line (for example ATCC, reference CCL 81) according to the standard techniques that are well known to those skilled in the art. Mention may be made of the calcium phosphate technique (Maniatis et al., supra). However, other protocols enabling a nucleic acid to be introduced into a cell may also be employed, such as the DEAE dextran technique, electroporation, methods based on osmotic shocks, the microinjection of a selected cell or methods based on the use of liposomes.

The cells are cultured according to the standard conditions and are placed in a selective medium containing puromycin, 24 hours after transfection.

The expression of the genes of the E1 region is evaluated using various cell clones and the clone which is most productive, which may be used as a complementation line for the preparation of an adenovirus which is defective for the E1 function, such as the adenovirus detailed in Example 2, is determined. A complementation line containing a complementation element borne by pTG6557, pTG6558, pTG6564 or pTG6565 comprises no Ad5 sequences in common with such an adenovirus, and as such there is no risk of recombination.

EXAMPLE 5: Formation of a complementation line for all of the functions essential to the replication of an adenovirus

A vector is constructed comprising the whole of the Ad5 adenoviral genome with the exception of the 5' ITR, the 3' ITR and the encapsidation region.

The vector pTG6528 (described in Example 4) is digested with the enzymes *Pst*I and *Bgl*II. Between these sites there is inserted a DNA fragment, synthesized chemically according to the standard protocol, consisting of the oligonucleotides of the OTG5039 and OTG5040 (SEQ ID NO: 10 and 11). The oligonucleotide sequence is designed so as not to re-form the *Pst*I cloning site and to introduce an *Eco*RV site. pTG1639 is obtained, which is linearized by *Eco*RV digestion and ligated to an *Xba*I-*Bam*HI fragment whose ends are rendered blunt by treatment with Klenow DNA polymerase. This fragment carries the SV40 virus transcription termination signal. Any plasmid containing a signal surrounded by appropriate restriction sites may be used in this step.

The vector pTG1640 thus generated is digested with *Bam*HI and *Bgl*II, and the fragment carrying the cassette for the expression of the pac gene is introduced into the *Bgl*II site of the vector p PolyII-Sfi/Not-14* (Lathe et al., supra). pTG1641 is obtained. The latter is linearized with *Not*I and treated with Klenow DNA polymerase. The 0.276-kb *Bam*HI-*Sal*I fragment isolated from pBR322 (Bolivar et al., *supra*) also treated with Klenow DNA polymerase is introduced. This gives rise to pTG1643.

pTG1643 is linearized with *Xho*I, and an *Xho*I hybrid fragment containing a dimer of UAS elements from the Gal10 gene placed at 5' of the minimum promoter of the TK-HSV-1 gene. The UAS dimer is synthesized chemically using the oligonucleotides reported in SEQ ID NO:12 and 13. The DNA fragment thus generated is introduced at 5' of the minimum promoter of the TK-HSV1 gene. This promoter corresponds to the fragment proceeding from nucleotide 303 and terminating at nucleotide 450 of the sequence disclosed in the Genebank database under reference V00467 and into which has been introduced an *Xho*I site in the 3' position of said promoter. pTG1647 is obtained, in which the UAS Gal10-TK-HSV-1 hybrid promoter is inserted in the same

orientation as the cassette for the expression of the pac gene.

This construction, pTG1647, is used as a parent vector for introducing, between the *Pst*I and *Bam*HI sites, a fragment of the Ad5 genome, and more particularly the Ad5 fragment ranging from nucleotide 505 to nucleotide 35826.

In a first stage, pTG1647 is digested with *Pst*I and *Bam*HI and then ligated, on the one hand to the *Pst*I-*Cla*I fragment of pTG6552 (see Example 4) containing the portion of the Ad5 genome from nucleotides 505 to 918, and on the other hand to the *Cla*I-*Bam*HI fragment prepared from Ad5 genomic DNA and corresponding to the Ad5 sequence from nucleotide 918 to nucleotide 21562. The vector thereby obtained contains the 5' portion of Ad5 with the exception of the 5'ITR and the encapsidation region.

Separately, the 3' portion of the Ad5 genome is assembled in the vector p polyII-Sfi/Not-14* (Lathe et al., supra). The latter is linearized with *Bam*HI, and the *Bam*HI-*Avr*II fragment (nucleotides 21562 to 28752) isolated from the Ad5 genomic DNA and a PCR-generated fragment corresponding to the Ad5 genomic sequence of nucleotides 35463 to 35826. This fragment is generated by PCR using the Ad5 genomic DNA and the primers OTG5024 (SEQ ID NO:14) and OTG5025 (SEQ ID NO:15) containing a *Bam*HI site at 5'. The resulting vector is then digested with *Avr*II and the *Avr*II fragment isolated from the Ad5 genomic DNA and extending from positions 28753 to 35462 is inserted.

The vector generated in the preceding step is digested with *Bam*HI and the *Bam*HI fragment containing the adenoviral sequences is introduced into the *Bam*HI site of the vector described above containing the 5' portion of the adenoviral genome lacking the 5' ITR and the encapsidation region.

A vector is thus generated containing a complementation element for all of the functions of an adenovirus.

The complementation line is generated by transfection into a cell line according to the protocol described in the preceding example.

EXAMPLE 6: Formation of a complementation line for the E1 and E4 functions

The vector pTG1647 is digested with the enzymes *Pst*I-*Bam*HI, and 3 fragments are introduced into the vector thus treated:

- the *Pst*I-*Xba*I fragment of pTG6552 (Example 4) carrying the Ad5 sequences from nucleotide 505 to nucleotide 1339,
- the *Xba*I-*Sph*I fragment of pTG6552 carrying the Ad5 sequences from nucleotide 1340 to nucleotide 3665, and
- the *Sph*I-*Bam*HI fragment of pTG6554 (Example 4) carrying the Ad5 sequences from nucleotide 3665 to 4034 and a transcription termination signal.

The vector thereby obtained is cut with *Bam*HI, and the following three fragments are introduced into this site:

- a fragment digested with *Bam*HI-*Afl*II, generated by PCR, corresponding to the Ad5 sequence located between positions 32800 and 33104. The procedure used employs Ad5 genomic DNA as template and the primers OTG5078 (SEQ ID NO: 16) and OTG5079 (SEQ ID NO: 17),
- the *Afl*II-*Avr*II fragment isolated from Ad5 genomic DNA (nucleotides 33105 to 35463),
- the *Avr*II-*Bam*HI fragment generated by PCR using the primers OTG5024 and OTG5025 (see Example 5).

The vector thereby generated is introduced into a cell line according to the protocol described above, to form a complementation line.

SEQUENCE LISTING

SEQ ID NO: 1

TYPE: nucleic acid
LENGTH: 30 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: mutagenesis oligonucleotide (OTG4174) enabling the deletion of nucleotides 184 to 273 of the Ad5 encapsidation region.

GTGACGTCTT TGGTGTTTTC GCGGGAAAAC 30

SEQ ID NO: 2

TYPE: nucleic acid
LENGTH: 39 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: mutagenesis oligonucleotide (OTG4173) enabling the deletion of nucleotides 270 to 346 of the Ad5 encapsidation region.

GGCCATGGTC GCGGGAAAAC TGAAGGGACT TTGACCGTT 39

SEQ ID NO: 3

TYPE: nucleic acid
LENGTH: 31 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no

MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5021) for isolating a fragment of the Ad5 genome (nucleotides 4047 to 4614) and containing at the 5′ end a *Bam*HI site intended to facilitate the subsequent cloning steps.

GAACGGATCC CCAGACTCTG TTTGGATTTG G 31

SEQ ID NO: 4

TYPE: nucleic acid
LENGTH: 30 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5157) corresponding to nucleotides 4656 to 4639 of the Ad5 genome.

CCAGAAATAT CTTCGCCCAG GCCGCCGCCC 30

SEQ ID NO: 5

TYPE: nucleic acid
LENGTH: 32 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG4564) for amplifying the 5′ end of the Ad5 genome from which the 5′ ITR has been deleted; OTG4564 corresponds to nucleotides 100 to 120 of Ad5 and also contains, at its 5′ end, an *Eco*RI site intended to facilitate the subsequent cloning steps.

TCCGTGAATT CTAGTAGTGT GGCGGAAGTG TG 32

SEQ ID NO: 6

TYPE: nucleic acid
LENGTH: 23 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG4565) for amplifying the 5' end of the Ad5 genome from which the 5' ITR has been deleted; OTG4565 corresponds to nucleotides 831 to 814 of Ad5.

TCCAGTCCGG AGAACCGGGC GCC 23

SEQ ID NO: 7

TYPE: nucleic acid
LENGTH: 28 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5013) corresponding to nucleotides 505 to 522 of Ad5 and containing at the 5' end a *Pst*I site intended to facilitate the subsequent cloning steps.

TAACCTGCAG GAGTGCCAGC GAGTAGAG 28

SEQ ID NO: 8

TYPE: nucleic acid
LENGTH: 21 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear

ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5015) corresponding to nucleotides 3655 to 3675 of Ad5.

CAACGCGCAT GCCCCCATGG G 21

SEQ ID NO: 9

TYPE: nucleic acid
LENGTH: 31 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5014) corresponding to nucleotides 4034 to 4014 of Ad5 and containing at the 5′ end a *Bgl*II site intended to facilitate the subsequent cloning steps.

TAGGAGATCT GTTTTAAACC GCATTGGGAG G 31

SEQ ID NO: 10

TYPE: nucleic acid
LENGTH: 16 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: oligonucleotide (OTG5039) introducing an *Eco*RV cloning site.

TGCTGGATAT CAGTCA 16

SEQ ID NO: 11

TYPE: nucleic acid
LENGTH: 24 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: oligonucleotide (OTG5040) introducing an *Eco*RV cloning site.

GATCTGACTG ATATCCAGCA TGCA 24

SEQ ID NO: 12

TYPE: nucleic acid
LENGTH: 34 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: oligonucleotide corresponding to a UAS dimer of the Gal10 gene of *Saccharomyces cerevisiae*.

CGGAGTACTG TCCTCCGCGG AGTACTGTCC TCCG 34

SEQ ID NO: 13

TYPE: nucleic acid
LENGTH: 34 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: oligonucleotide corresponding to a UAS dimer of the Gal10 gene of *Saccharomyces cerevisiae*.

CGGAGGACAG TACTCCGCGG AGGACAGTAC TCCG 34

SEQ ID NO: 14

TYPE: nucleic acid
LENGTH: 20 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5024) corresponding to nucleotides 35457 to 35476 of Ad5.

CTCCTGCCTA GGCAAAATAG 20

SEQ ID NO: 15

TYPE: nucleic acid
LENGTH: 32 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5025) corresponding to nucleotides 35826 to 35807 of Ad5 and containing at the 5′ end a *Bam*HI site intended to facilitate the subsequent cloning steps.

GCAGATGGAT CCGGGCGGAG TAACTTGTAT GT 32

SEQ ID NO: 16

TYPE: nucleic acid
LENGTH: 31 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear

ANTISENSE: no
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5078).

GTCGCGGATC CGTTATGTTT CAACGTGTTT A 31

SEQ ID NO: 17

TYPE: nucleic acid
LENGTH: 20 nucleotides
STRANDEDNESS: single
TOPOLOGY: linear
ANTISENSE: yes
MOLECULE TYPE: DNA

PROPERTIES: PCR primer (OTG5079).

ACATGAACTT AAGCGAGCTG 20

Claims

1. Adenovirus which is defective for replication and which is selected from:

(a) an adenovirus which is derived from a natural adenovirus in that it lacks:

(i) at least the coding sequences of the E1B region, and/or

(ii) at least a part of the E4 region;

(b) an adenovirus which is derived from a natural adenovirus other than a human type 5 adenovirus in that it lacks a part of the encapsidation region.

2. Adenovirus which is defective for replication and which is selected from:

(a) an adenovirus which is derived from a natural adenovirus in that it lacks:

(i) at least the coding sequences of the E1B region, and/or

(ii) at least a part of the E4 region;

(b) an adenovirus which is derived from a human type 5 adenovirus in that it lacks:

(i) a part of the encapsidation region extending from nucleotide 270 to nucleotide 346 of the sequence as disclosed in the Genebank database under reference M73260, or

(ii) a part of the encapsidation region extending from nucleotide 184 to nucleotide 273 of the sequence as disclosed in the Genebank database under reference M73260.

3. Adenovirus according to Claim 2, derived from a

human type 5 adenovirus, [lacuna] in that it lacks:

(i) at least the coding sequences of the E1B region, with said deletion terminating at nucleotide 4047 of the sequence as disclosed in the Genebank database under reference M73260; and/or

(ii) all or part of the E4 region, said E4 region being located between nucleotides 32800 and 35826 of the sequence disclosed in the Genebank database under reference M73260; and/or

(iii) a part of the encapsidation region:

- extending from nucleotide 270 to nucleotide 346 of the sequence as disclosed in the Genebank database under reference M73260, or

- extending from nucleotide 184 to nucleotide 273 of the sequence as disclosed in the Genebank database under reference M73260.

4. Adenovirus according to one of Claims 1 to 3, also lacking all or part of the E1A region.

5. Adenovirus according to one of Claims 1 to 4, also lacking all or part of the E3 region.

6. Adenovirus according to Claim 5, lacking the part of the E3 region which does not comprise the sequences coding for the gp19kDa protein; said sequences coding for the gp19kDa protein being placed under the control of appropriate elements which enable them to be expressed.

7. Adenovirus according to one of Claims 1 to 6, also lacking all or part of the E2 region.

8. Adenovirus according to one of Claims 1 to 7, also lacking all of the genes coding for the early and

late proteins but comprising, nevertheless, the 5′ and 3′ ITRs and all or part of the encapsidation region of said adenovirus.

9. Adenovirus according to one of Claims 1 to 8, also containing an exogenous nucleotide sequence.

10. Adenovirus according to Claim 9, in which the exogenous nucleotide sequence comprises one or more genes of interest, said genes of interest being placed under the control of elements suitable for their expression.

11. Adenovirus according to Claim 10, in which (one of) the gene(s) of interest consist(s) of the human gene coding for the CFTR protein.

12. Adenovirus according to Claim 10, in which (one of) the gene(s) of interest consists of the thymidine kinase gene of type-1 herpes simplex virus.

13. Adenovirus according to one of Claims 1 to 12, also comprising a gene coding for a transcription trans-activating protein.

14. Adenovirus according to Claim 13, in which said gene encoding a transcription trans-activating protein consists of the gene coding for the *Saccharomyces cerevisiae* Gal4 protein.

15. Adenovirus according to one of Claims 1, 2 and 4 to 14, derived from a natural adenovirus selected from a canine adenovirus, an avian adenovirus and a human adenovirus.

16. Adenovirus according to one of Claims 2 to 14, derived from a human type 5 adenovirus.

17. Eukaryotic cell containing an adenovirus according to one of Claims 1 to 16.

18. Complementation line consisting of a cell line containing a complementation element comprising at least a portion of the E1 region of a natural adenovirus and lacking the 5′ ITR sequence; said complementation element being either integrated in the genome or inserted into a vector.

19. Complementation line according to Claim 18, in which said complementation element also lacks the

promoter of the E1A region; said promoter of the E1A region being replaced with an appropriate non-adenoviral promoter.

20. Complementation line according to Claim 19, in which said appropriate non-adenoviral promoter consists of a promoter which is inducible by a protein which trans-activates transcription.

21. Complementation line according to Claim 20, in which said promoter which is inducible by a protein which trans-activates transcription consists of a promoter which is inducible by the *Saccharomyces cerevisiae* Gal4 protein.

22. Complementation line according to one of Claims 18 to 21, in which said complementation element comprises at least a part of the E1 region of a human type 5 adenovirus selected from:

(i) the region, or a homologous region, proceeding from nucleotide 100 and terminating at nucleotide 5297 of the sequence as disclosed in the Genebank database under reference M73260, and

(ii) the region, or a homologous region, proceeding from nucleotide 100 and terminating at nucleotide 4034 of the sequence as disclosed in the Genebank database under reference M73260, and

(iii) the region, or a homologous region, proceeding from nucleotide 505 and terminating at nucleotide 4034 of the sequence as disclosed in the Genebank database under reference M73260; said region being placed under the control of the elements which are required for its expression.

23. Complementation line according to one of Claims 18 to 22, in which said complementation element also

comprises at least a part of the E4 region of a natural adenovirus.

24. Complementation line according to Claim 23, in which said complementation element comprises at least a part of the E4 region of a human type 5 adenovirus; said E4 region proceeding from nucleotide 32800 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260.

25. Complementation line according to one of Claims 18 to 24, in which said complementation element also comprises at least a part of the E2 region of a natural adenovirus.

26. Complementation line according to Claim 25, in which said complementation element comprises the whole of the genome of a natural adenovirus apart from the encapsidation region and the 5' ITR of said adenovirus.

27. Complementation line according to Claim 26, in which said complementation element comprises the part of the genome of a human type 5 adenovirus proceeding from nucleotide 505 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260.

28. Complementation line according to Claim 27, in which said part of the genome of a human type 5 adenovirus, proceeding from nucleotide 505 and terminating at nucleotide 35826 of the sequence as disclosed in the Genebank database under reference M73260, is placed under the control of an appropriate non-adenoviral promoter.

29. Complementation line according to one of Claims 18 to 28, in which said complementation element also comprises a gene coding for a selectable marker.

30. Complementation line according to Claim 29, in which said gene coding for a selectable marker consists of the gene coding for puromycin acetyltransferase.

31. Complementation line according to Claim 30, in which the gene coding for puromycin acetyltransferase is placed under the control of the E2A adenoviral

promoter.

32. Complementation line according to one of Claims 18 to 31, consisting of a pharmaceutically acceptable cell line selected from the Vero, BHK, A549, MRC5, WI38 and CHO lines.

33. Method for preparing a defective adenovirus according to one of Claims 1 to 16, according to which:

- said defective adenovirus is introduced into a complementation line according to one of Claims 18 to 32,

- said complementation line is cultured according to suitable conditions for permitting the production of said defective adenoviruses, and

- said defective adenoviruses are recovered in the culture supernatant of said line.

34. Therapeutic or prophylactic use of a defective adenovirus according to one of Claims 1 to 16, a eukaryotic cell according to Claim 17 or a complementation line according to one of Claims 18 to 32.

35. Pharmaceutical composition comprising as therapeutic or prophylactic agent a defective adenovirus according to one of Claims 1 to 16, a eukaryotic cell according to Claim 17 or a complementation line according to one of Claims 18 to 32, in combination with a vehicle which is acceptable from a pharmaceutical standpoint.

Novel defective adenoviruses and corresponding complementation lines

Transgene S.A.

Abstract

The present invention relates to novel defective adenoviruses for the transfer and expression of an exogenous nucleotide sequence in a host cell or organism. The invention also relates to novel complementation lines and to the method for preparing these novel defective adenoviruses, as well as to their therapeutic use and to a pharmaceutical composition containing them.